

Self-Protective Mechanism Awakened by Glutamate in Retinal Ganglion Cells

ETI YOLES, IGOR FRIEDMANN, RINA BAROUCH, Yael SHANI, and
MICHAL SCHWARTZ

ABSTRACT

The progression of degeneration in chronic optic neuropathies or in animal models of optic nerve injury is thought to be caused, at least in part, by an increase in glutamate to abnormally high concentrations. We show here that glutamate, when injected in subtoxic amounts into the vitreal body of the rat eye, transduces a self-protecting signal that renders the retinal ganglion cells resistant to further toxicity, whether glutamate-derived or not. This neuroprotective effect is attained within 24 h and lasts at least 4 days. Western blot analysis of rat retinas revealed increased amounts of bcl-2 four days after injection of glutamate in either subtoxic or toxic (120 nmol) amounts, but not after saline injection. The effects of intravitreal glutamate or saline injection on the secretion of neurotrophins by retinal ganglion cells was evaluated in rat aqueous humor 6 h, 1 day, and 4 days after injection. Nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 showed similar kinetic patterns in all of the eyes; that is, they increased to a peak 1 day after the injection and returned to normal by day 4. However, increased amounts of the neurotrophin receptor TrkA within the retinal ganglion cell layer and nerve fiber layer were detected 1 day after injection of glutamate in either toxic or subtoxic amounts, but not after saline injection. This finding points to the possible involvement of neurotrophin receptors in regulation of the cellular responses to glutamate challenge. Identification of the intracellular signals that trigger the glutamate-induced self-protective mechanism would shed light on the genetic balance needed for survival, and guide the development of drugs for the up-regulation of desired genes and their products.

Key words: glaucoma; glutamate; neuroprotection; optic neuropathy; retinal degeneration; visual evoked potential

INTRODUCTION

GLUTAMATE is the most abundant neurotransmitter in the mammalian central nervous system (CNS) in general and in the retina in particular. The physiological concentration of this amino acid is 1 μ M extracellularly and up to several millimolar intracellularly (Coyle and Puttfarcken, 1993). Above this extracellular concentra-

tion, glutamate becomes cytotoxic to neurons via both receptor mediation and the oxidative glutamate toxicity pathway. Enzymatic degradation does not occur extracellularly, but glutamate released from presynaptic structures is taken up by at least one of its three known transporters and metabolized intracellularly by a glutamate-degrading enzyme. Excitatory amino acid transporters that bind glutamate are distributed in Müller cells,

astrocytes, horizontal cells, amacrine cells, ganglion cells, and bipolar cells (Kanai et al., 1993). Nevertheless, a transient excess of unbound glutamate may occur in areas dense in glutamatergic terminals, causing neuronal and synaptic damage.

Recent studies have linked the progression of neuronal degeneration after chronic or acute CNS injury to increased glutamate concentrations in the extracellular milieu (Dreyer and Grosskreutz, 1997; Faden and Salzman, 1992; Rowland, 1994). This is true whether the degeneration is initiated in the cell bodies or in the axons. In the case of optic neuropathies, for example, irrespective of their etiology, an increase in glutamate is detected in the extracellular milieu of the cell bodies (the retinal ganglion cells; RGCs) of degenerating optic nerve fibers, mainly in the vitreous and aqueous humor (Dreyer et al., 1996; Yoles and Schwartz, 1998b).

In view of the ubiquity of glutamate in general and in the visual system in particular (Marc et al., 1990), a transient increase in extracellular glutamate may occur at any time, and particularly under pathological conditions. It is therefore reasonable to assume the existence of an endogenous mechanism that can counteract glutamate toxicity without diminishing its essential excitatory effect as a neurotransmitter. It is likely that such a mechanism operates at any level of glutamate exposure, and that the intracellular balance between the survival signals and the death signals transduced by glutamate will affect the fate of the cell.

In seeking such signals, we assumed that if they existed they would probably be detectable upon exposure to glutamate at concentrations that are higher than physiological but not yet cytotoxic. Using the rat visual system as an experimental model in the present study, our search yielded evidence for the existence of a glutamate-induced protective mechanism which can counteract, to some extent, the effects of extracellular cytotoxicity.

MATERIALS AND METHODS

Surgical Procedure

Animals. Rats were handled according to the ARVO resolution on the use of animals in research. Male Sprague-Dawley (SPD) rats, weighing 250–300 g, from the Weizmann Institute of Science animal house were anesthetized with Vetalar (ketamine, 60 mg/kg) and Rompun (xylazine, 12 mg/kg), both administered intramuscularly. Prior to tissue excision the rats were killed by an overdose of sodium pentobarbitone (170 mg/kg intraperitoneally; CTS Chemical Industries, Tel-Aviv, Israel).

Intravitreal injections. The right eye of each anesthetized rat was punctured with a 27-gauge needle in the

upper part of the sclera, and a Hamilton syringe was inserted as far as the vitreal body. A solution of glutamate in various amounts between 20 and 240 nmol or 100 nmol ammonium-fer(II) sulfate hexahydrate (Merck, Germany) in saline (total volume of 1 μ L) was injected. The final concentration in the vitreous was calculated assuming a vitreal volume of 50 μ L.

Retrograde Labeling and Counting of Retinal Ganglion Cells

After 6 h, 1 day, or 4 days, the right optic nerves of rats were exposed intraorbitally and solid crystals (0.2–0.4 mm diameter) of the fluorescent lipophilic dye 4-(4-didecylamino)styryl)-*N*-methylpyridinium iodide (Molecular Probes, Europe BV, Leiden, The Netherlands) (4-Di-10-Asp) were deposited 2 mm from the eyeball. As shown previously in this model (Yoles and Schwartz, 1998a), only axons that are viable are capable of retrograde transfer of the dye, and counting of labeled RGCs 5 days after dye application gives reproducible results. The dye application procedure itself has no negative effect during the period until retinal excision. Five days after dye application, rats were given a lethal dose of pentobarbitone. From each eye the retina was detached, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined by fluorescence microscopy. Labeled RGCs were counted from six randomly selected fields (0.78 mm² per field) in each retina, averaged, and the mean number of RGCs per mm² was calculated.

Immunoblot (Western Blot) Analysis

At the indicated time periods after intravitreal injection of glutamate or saline the rats were killed, their right eyes were enucleated, and the retinas were quickly detached, homogenized with a lysis buffer containing Tris (10 mM, pH 7.2), NaCl (150 mM), Triton X-100 (1%), EDTA (5 mM), sodium dodecyl sulfate (0.1%), sodium deoxycholate (1%), aprotinin (25 g/mL), leupeptin (25 g/mL), pepstatin (5 g/mL), and phenylmethylsulfonyl-fluoride (PMSF; 1 mM) at 4°C, and the supernatant was collected. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% gel) followed by blotting onto a nitrocellulose membrane for 2 h at 200 mA (in Tris-glycine). The membrane was incubated overnight at 4°C with phosphate-buffered saline (PBS) containing 5% (vol/vol) skim milk, incubated with a monoclonal antibody to bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 5% skim milk for 2 h at 37°C, and washed three times for 20 min in PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween-20). It was then incubated with horseradish peroxidase-conjugated goat anti-rabbit

GLUTAMATE EVOKES SELF-PROTECTION IN RETINAL GANGLION CELLS

IgG (Jackson ImmunoResearch, West Grove, PA) in PBS containing 5% skim milk for 1 h at room temperature and washed three times for 20 min in PBS containing 0.05% Tween-20. Immunoreactive bands were visualized by the enhanced chemiluminescence method (ECL; Amersham, Buckinghamshire, U.K.).

Immunohistochemistry

One day after intravitreal injection of glutamate or saline the rats were killed, their eyes were dissected out, and a small cut was made in the cornea of each eye to improve fixation of the retina. The tissues were fixed overnight at 4°C in 4% paraformaldehyde and 5% glucose. Eyes were embedded in Tissue-Tek (Sakura, Torrance, CA). Cryostat sections (10 μ m thick) of the retina were placed on gelatin-coated slides, washed twice in double-distilled water, and incubated for 5 min in PBS containing 0.05% Tween-20 and for 30 min in PBS containing 3% fetal calf serum, 2% bovine serum albumin, and 1% Triton X-100 (Sigma, St. Louis, MO). They were then incubated for 1 h at room temperature with mouse monoclonal antibodies to HSP72 (StressGen Biotechnologies, Victoria, Canada) or overnight at 4°C with rabbit monoclonal antibodies to the neurotrophin receptor TrkA (Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies were diluted in PBS containing 3% fetal calf serum and 2% bovine serum albumin. The sections were washed three times with PBS and Tween-20 (0.05%) and incubated with rhodamine-conjugated goat anti-mouse

IgG for 1 h at room temperature. After further washing with PBS containing Tween-20, the sections were mounted with glycerol containing 1,4-diazobicyclo-(2,2,2)octane to reduce quenching of fluorescence, and viewed under a Zeiss fluorescence microscope.

Enzyme-Linked Immunosorbent Assay

Samples of aqueous humor (20- μ L aliquots) were collected from the rats 6 h, 1 day, or 4 days after intravitreal injection of saline or glutamate in the indicated amounts and immediate frozen on dry ice until analyzed. Concentrations of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin (NT)-3 in the aqueous humor were determined. Neurotrophin concentrations were determined by the use of sandwich enzyme-linked immunosorbent assay (ELISA) kits (Promega, Madison, WI) and comparison with a standard (absorbance measured at 450 nm using an ELISA reader).

RESULTS

Concentration-Dependent Effect of Glutamate on Retinal Ganglion Cell Survival

Adult rats were injected intravitreally with glutamate at various dosages (between 20 and 120 nmol, corresponding to vitreal concentrations of approximately 0.4–2.4 mM), and RGC survival was quantified by counting the retrogradely labeled cells. After 4 days, there was

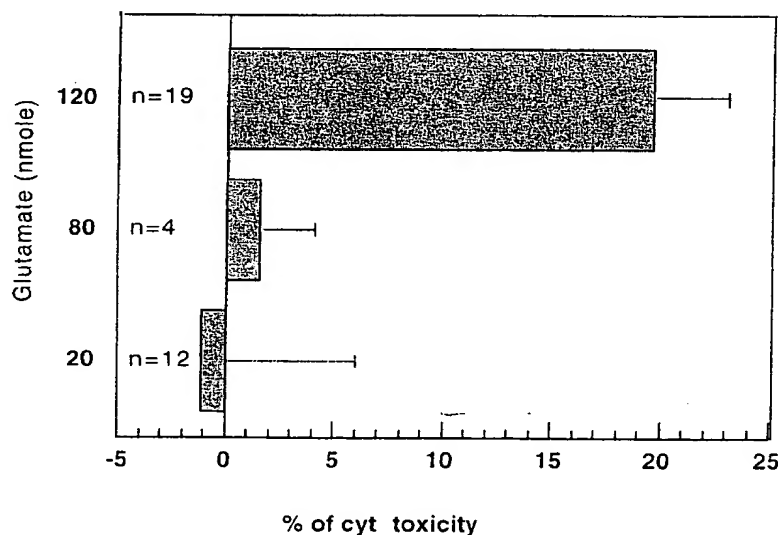


FIG. 1. Dose-dependent effect of intravitreal glutamate injection on retinal ganglion cell survival. Glutamate was injected intravitreally, reaching the final concentrations indicated in the figure. Eyes injected with PBS ($n = 7$) served as controls. The amount of cytotoxicity was calculated as a percentage of that in the control group. Results are presented as means \pm SEM. The effect of glutamate dosage on RGC survival was significant (ANOVA, $F = 3.68$, $p = 0.01$).

a 20% loss of RGCs treated with 120 nmol glutamate but no loss with glutamate treatment of 20 or 80 nmol (Fig. 1). This finding prompted us to examine the possibility that the lower dosages which, though higher than normal physiological amounts are obviously not toxic, might affect the cells in a way that influences their response to subsequent glutamate exposure. Accordingly, we examined the response of the neurons to a second injection of glutamate (120 nmol). Surprisingly, the fact that these cells were previously injected with low-dose glutamate (20 nmol) not only failed to make them more susceptible to subsequent glutamate toxicity, but even protected them from it. Thus, glutamate at 120 nmol, a dosage that was toxic to RGCs of naive retinas, had no toxic effect if administered 4 days after the 20 nmol dose (Fig. 2).

The protective effect of the earlier injection was not seen if the interval between the two injections was only 6 h. When the interval was prolonged to 1 day or 4 days, however a significant neuroprotective effect was seen ($p < 0.03$ or $p < 0.002$, respectively; Fig. 2). By 11 days

after injection the protective effect, although still seen, was not significant when compared to the saline-injected group. These findings suggested that 20 nmol glutamate is not only not toxic, but also does not render the cells more sensitive to further glutamate toxicity. On the contrary, it apparently activates an intracellular and/or extracellular mechanism that makes the cells more resistant. The results further suggested that the observed protective effect is time-dependent.

To determine whether the induced resistance is only to glutamate toxicity, we replaced the second glutamate injection by an injection of ammonium ferrous sulfate (Fig. 3). The toxicity of this compound derives from the reaction of ferrous ions to metal-binding sites on the protein, producing an active oxygen species (Stadtman, 1990). Our results showed that concentrations of ferrous ions that were toxic to naive RGCs were not toxic to RGCs preexposed to low-dose glutamate (20 nmol; final vitreal concentration 0.4 mM).

The above findings suggested that low-dose glutamate

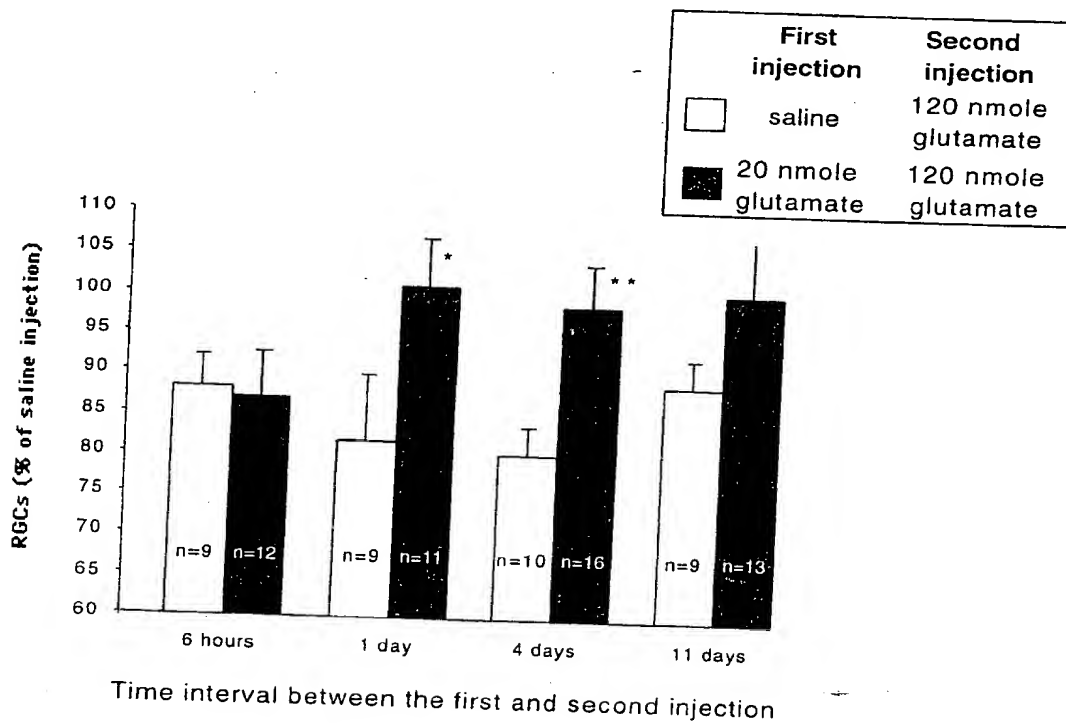


FIG. 2. Effect of intravitreal injection of 20 nmol glutamate on the subsequent toxic effect of 120 nmol intravitreal glutamate injected at different times. Each pair of bars represents the effect of 120 nmol glutamate injected after injection of saline (left bar) or of 20 nmol glutamate (right bar), expressed as a percentage of the number of RGCs observed in retinas after two injections of saline at the same time interval. Results are presented as means \pm SEM. Each group contained nine to 11 rats. The effect of prior exposure to glutamate relative to saline, where the interval between the first and second injections was 1 day or 4 days, was significant (one-tail t test, $p < 0.04$ and $p < 0.002$, respectively). When the time interval was 11 days, the difference was not significant ($p < 0.09$).

GLUTAMATE EVOKES SELF-PROTECTION IN RETINAL GANGLION CELLS

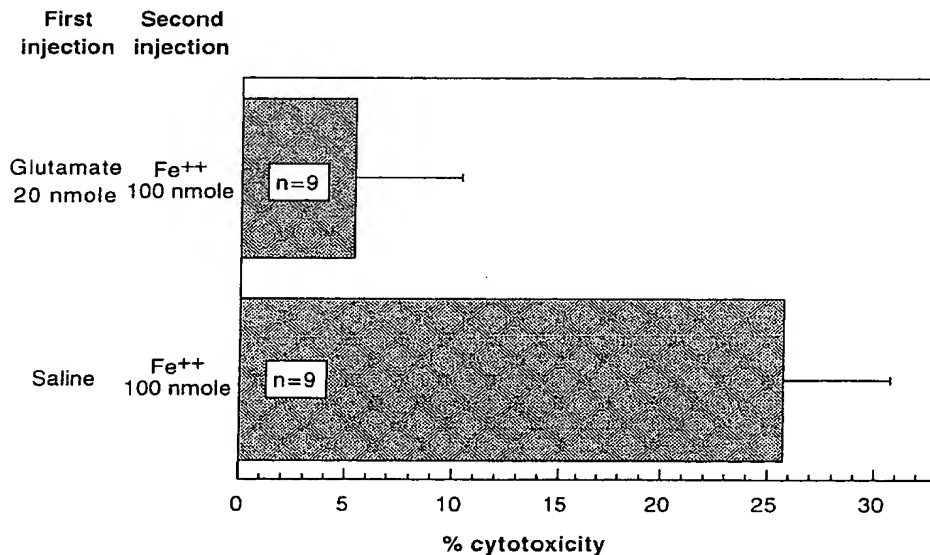


FIG. 3. Intravitreal injection of glutamate (20 nmol) protects retinal ganglion cells from toxicity mediated by free radicals. Ferrous ammonium sulfate (100 nmol) was injected intravitreally 4 days after injection of glutamate (20 nmol) or saline. Results are presented as means \pm SEM of the percentage of cytotoxicity obtained relative to that in rats injected twice with saline ($n = 5$ for each group). The difference between the two groups was significant (Student's t test, $p = 0.02$).

renders the RGCs more resistant to toxicity, and not only to glutamate toxicity. The increased resistance might result, for example, from activation of an efficient buffering mechanism for glutamate, possibly via the activation of enzymes such as glutamate synthase or of glutamate transporters by Müller cells. Measurements of glutamine synthase activity in the retina showed that it was not affected by intravitreal injections of saline or glutamate at any of the concentrations tested (data not shown).

Another possibility is that the resistance mechanism does not operate at the level of glutamate buffering. Low-dose glutamate might trigger, as a self-repair or self-protective mechanism, intracellular signal transduction in the retina and/or extracellular changes which, at high glutamate concentrations, are overridden by destructive mechanisms.

Cellular and Molecular Mechanisms of Self-Protection Awakened by Subtoxic Glutamate

In an attempt to determine whether the increased cellular resistance conferred by subtoxic doses of glutamate is related to mechanisms of self-protection, we assayed the amounts of NGF, BDNF, and NT3 secreted into the rat aqueous humor. Aqueous humor was collected from rat eyes at different times after intravitreal injection, and the concentration of each neurotrophin was determined

by ELISA and compared with the normal concentration in non-injected eyes. In general, the effects observed with glutamate at both dosages and with saline were similar (Fig. 4). In all tested neurotrophins, a gradual increase began 6 h after the injection, peaked at 24 h, and returned to normal or below normal values by day 4. Although some differences in the neurotrophin responses were observed, no conclusions could be drawn concerning neurotrophin specificity to glutamate in general or to a specific dosage in particular.

Since the increase in neurotrophins after intravitreal injection appeared to be nonspecific (i.e., it was induced by both glutamate and saline), we examined the possibility that an effect was induced specifically by glutamate on the neurotrophin receptor Trk-A. Immunohistochemical staining demonstrated the presence of Trk-A in the RGC layer and nerve fiber layer of eyes injected 24 h earlier with either toxic or subtoxic amounts of glutamate (Fig. 5). No Trk-A was detected after saline injection or in normal, noninjected eyes.

Neurotrophins and their receptors are part of the repertoire of extracellular signals that can enhance cell resistance and survival under conditions of stress. Bcl-2, an intracellular protein, also affects cell resistance and survival under stress. Glutamate at both dosages caused an increase in bcl-2, whereas saline did not (Fig. 6). Another protein that participates in intracellular self repair is the inducible heatshock protein (hsp-72). Retinas exposed to

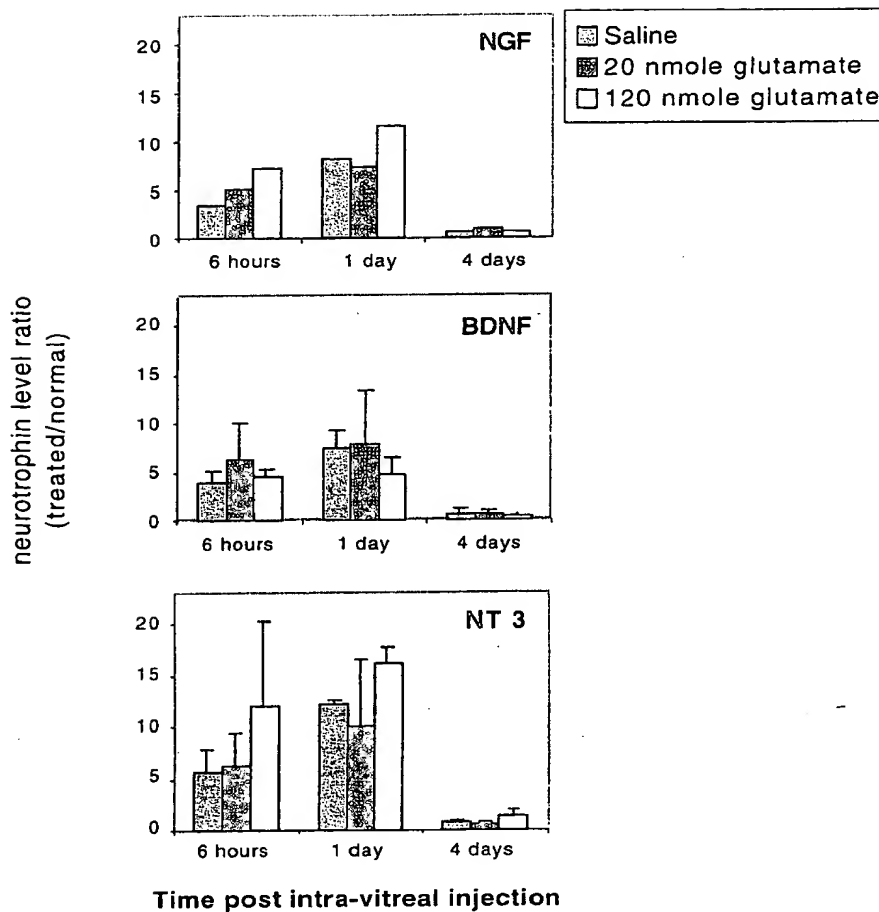


FIG. 4. Time-dependent secretion of neurotrophins into the aqueous humor of the rat eye as a consequence of glutamate or saline injection. Samples of aqueous humor (pooled from four eyes at each time point) were obtained 6 h, 1 day, or 4 days after intravitreal injection of saline, 20 nmol glutamate, or 120 nmol glutamate. Aqueous humor from normal (noninjected) eyes served as a control (uninjured eye). The amounts of NTs were determined by ELISA and were calculated relative to the control. In both saline-injected and glutamate-injected samples, all tested NTs began to increase by 6 h after the injection, peaked at 1 day, and returned to normal or below normal by day 4. Values of BDNF and NT3 are means \pm SD of three and two experiments, respectively (each carried out in triplicate).

toxic or subtoxic glutamate concentrations show no change in hsp-72 (data not shown).

These results imply that glutamate, irrespective of its concentration, triggers a survival signal, but that the fate of the cell will be determined by additional glutamate-triggered signals which, depending on the glutamate concentration, may override the survival signal.

DISCUSSION

The results of this study showed that glutamate, in amounts that are higher than physiological but not yet toxic, has a neuroprotective effect on RGCs. Rat retinas

that were exposed to these intermediate amounts of glutamate became more resistant to subsequent exposure to glutamate at toxic concentrations.

Glutamate is the major excitatory neurotransmitter in the retina. Photoreceptors, bipolar cells, and ganglion cells in the retina are all glutamate immunoreactive (Connaughton et al., 1999; Davanger et al., 1991; Jojich and Pourcho, 1996; Marc et al., 1990). Glutamate activity is therefore essential for the proper functioning of the visual pathway, and blocking of its activity by the use of antagonists can be harmful. On the other hand, increased amounts of glutamate exert a receptor-mediated (Bruno et al., 1993; Olney, 1994a,b; Sucher et al., 1997) and oxidative (Nakao and Brundin, 1998) toxicity.

GLUTAMATE EVOKES SELF-PROTECTION IN RETINAL GANGLION CELLS

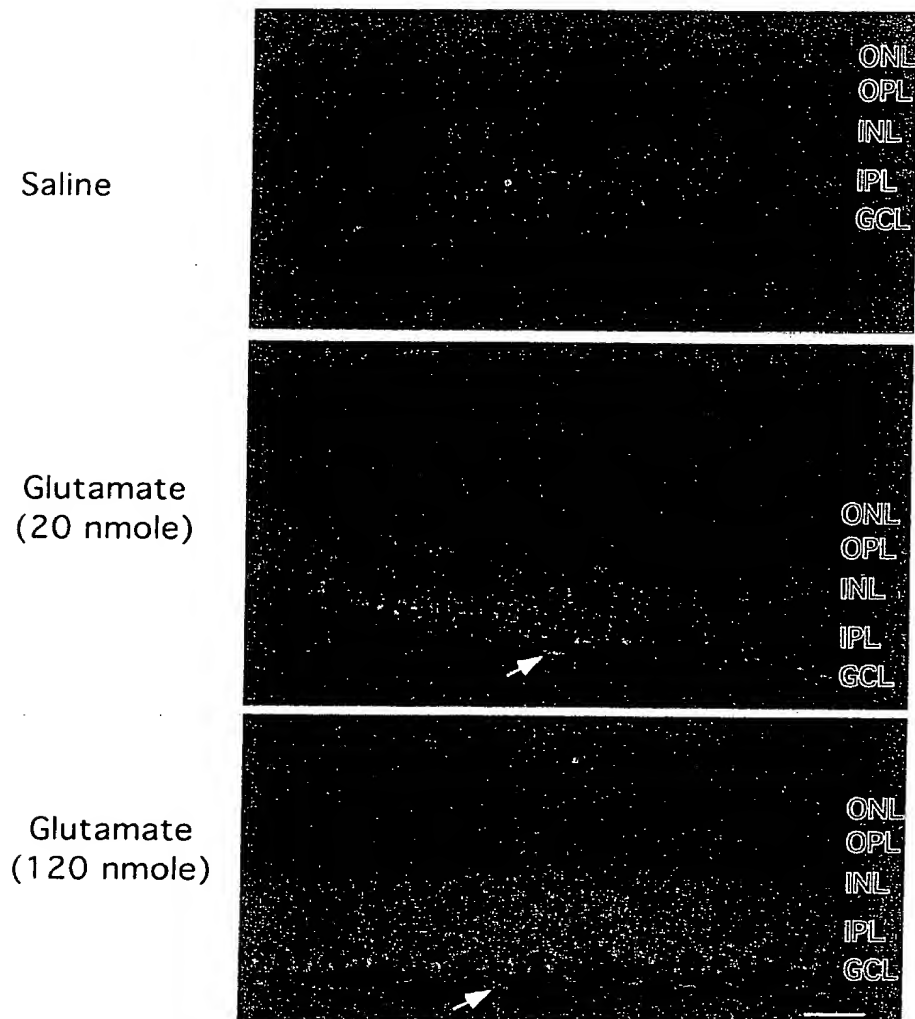


FIG. 5. Photomicrographs showing TrkA-immunoreactivity in retinas injected with saline, 20 nmol glutamate, or 120 nmol glutamate. Transverse retinal sections were labeled with antibodies directed against TrkA. Immunoreactivity can be seen in the ganglion cell layer (GCL) and nerve fiber layer (arrows) of glutamate-injected retinas but not of saline-injected retinas. Bar = 35 μ m.

Two classes of glutamate receptors have been identified: (1) ionotropic glutamate receptors, which directly gate ion channels, and (2) metabotropic glutamate receptors, which may be coupled to an ion channel or other cellular functions via an intracellular second-messenger cascade. The former receptors were recently shown to have some neuroprotective effect in traumatic injury (Faden et al., 1997), oxidative stress (Sagara and Schubert, 1998), and NMDA-induced excitotoxicity (Mukherjee et al., 1999; Pizzi et al., 1993, 1996). Some recent reports describe the involvement of the ionic glutamate receptor, NMDA, in neuroprotection (Grabb and Choi,

1999; Rocha et al., 1999). It was suggested that the NMDA receptor activates the TrkB receptor via an autocrine loop of BDNF, resulting in neuronal survival (Aliaga et al., 1998; Marini et al., 1998; Tsukahara et al., 1998). The protective effect of glutamate via activation of metabotropic and/or ionic glutamate receptors, as described above, may reflect a glutamate-induced regulation of neuronal survival and death. These short-term effects, however, can hardly explain the present finding of long-term protection against glutamate excitotoxicity and free-radical toxicity achieved by intravitreal injection of glutamate in subtoxic amounts. This notion is further

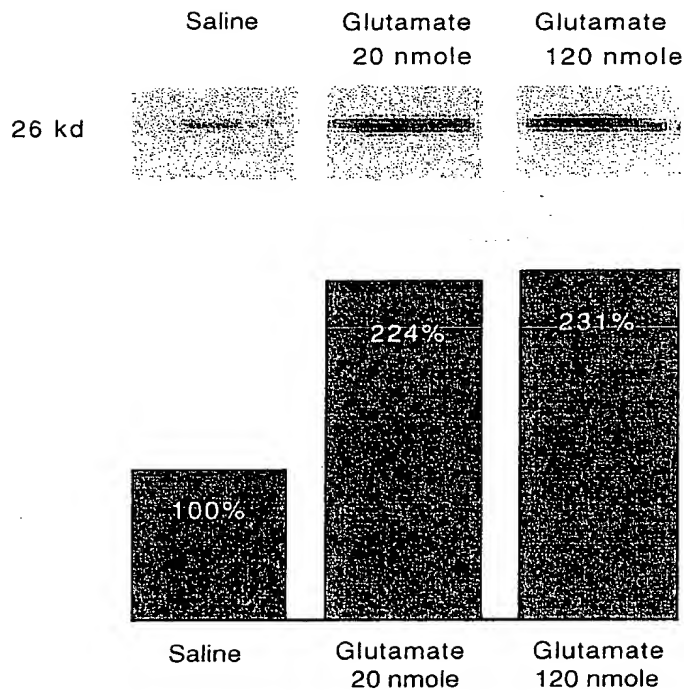


FIG. 6. Intravitreal glutamate injection increases the amount of bcl-2 in the retina. Representative results of immunoblotting for bcl-2 are shown. The experiment was repeated four times, with similar results.

supported by the observed lack of effect of exposure of glutamate, in any of the tested amounts, on the activity of the enzyme glutamine synthase. Moreover, the fact that the protective effect of glutamate requires at least 24 h to develop fully and lasts for at least 4 days suggested that it involves fundamental downstream molecular changes, which increase the ability of the neurons to cope with stress. Any stressful condition might initiate this active process of intracellular molecular changes that increase neuronal resistance. The fate of the neurons will then be a function of the glutamate concentrations. One possible way in which subtoxic amounts of glutamate may render the RGCs better able to cope with stressful conditions is via neurotrophin-related activity.

Neurotrophins (NTs) are biologically active proteins that promote neuronal survival through receptor-mediated processes and are thought to participate in nervous system development, maintenance, and response to trauma. NGF, BDNF, NT-3, and NT-4/5 have all been shown to enhance neuronal survival *in vitro* and *in vivo*. For example, NGF is required for the survival of sympathetic and some sensory and cholinergic neuronal populations (Levi Montalcini, 1987). BDNF prevents the death of motoneurons in newborn rats after nerve transection (Sendtner et al., 1992) and rescues spinal cord

motoneurons from axotomy-induced cell death (Yan et al., 1992). NT-4/5 increases RGC survival and neurite outgrowth in adult rats (Cohen et al., 1994; Sawai et al., 1996). The responsiveness of target cells to a given NT is governed by the expression of two classes of NT receptors: the low-affinity receptor p75, which binds all NTs with similar affinity (Chao, 1994), and the high-affinity tyrosine kinase receptors of the Trk family, which interact with NTs in a specific manner. A search for the binding partners of various Trk family members revealed that NGF is the preferred ligand for TrkA, BDNF and NT4/5 for TrkB, and NT3 for TrkC (Friedman and Greene, 1999). A number of studies have suggested that various pathologies of the visual system might be attributable, at least in part, to the distribution (Nag and Wadhwa, 1999; Caminos et al., 1999) and regulation (Pease et al., 2000; Johnson et al., 2000; Hu et al., 1999) of NTs and their receptors in the retina.

In the present work, injections of glutamate and of saline were both found to lead to a transient increase in NTs secreted into the aqueous humor. This would argue against increasing NTs as a way to induce neuronal resistance to glutamate-induced toxicity. In contrast, Trk-A expression was increased after injection of glutamate but not of saline. In view of these two findings, we suggest

GLUTAMATE EVOKES SELF-PROTECTION IN RETINAL GANGLION CELLS

that the increase in NT secretion is part of the self-repair mechanism that is activated in cases of trauma. This suggestion would also seem to be in line with the reported increase in BDNF mRNA following mechanical damage to the retina (Sakai et al., 1999). However, the increase in TrkA observed here may reflect a receptor-mediated specific effect of glutamate, resulting in activation of an intracellular signal that increases TrkA in both RGCs and nerve fibers. Regulation of the amounts of TrkA receptor mRNA appears to be induced by excitatory amino acid receptor agonists in the striatum (Canals, 1999), starting 10–12 h after stimulation. An increase in Trk-A was also found to play a role in regeneration of the injured optic nerve (Camino et al., 1999). Where the dosage of glutamate is high, this beneficial effect may be counteracted by glutamate-triggered destructive processes.

One way in the NTs can affect neuronal survival is via regulation of intracellular survival genes, such as bcl-2. The finding that CREB is a major mediator of neurotrophin-induced transcription (Ginty et al., 1994; Finkbeiner et al., 1997), and that it regulates bcl-2 in vivo, suggests that bcl-2 or a close family member might mediate neurotrophin-dependent survival (Finkbeiner, 2000). The glutamate-induced changes in bcl-2 content observed in this study might be typical of changes that occur in any retinal cells, including the RGCs, in response to glutamate challenge. The bcl-2 family of proteins are regulators that block programmed cell death (Chao and Korsmeyer, 1998; Korsmeyer, 1999; McDonnell et al., 1996). In the nervous system, overexpression of bcl-2 protects neurons from death induced by various traumatic insults (Garcia et al., 1992; Mah et al., 1993; Zhong et al., 1993). We showed here that glutamate in sub-toxic amounts causes an increase of about twofold in the amounts of bcl-2 in the retina. The same effect was also found, however, in retinas that were injected with glutamate in toxic amounts. We suggest that the increase in bcl-2 is an adaptive cellular response to stress, but that in cases of severe insult this response is insufficient to override death signals. Among such death signals are the death-associated genes of the bcl-2 family. In patients with Parkinson's disease, for example, bcl-2 was found to be up-regulated in the basal ganglia, a region subjected to stress that might be of many years' standing (Marshall et al., 1997). The nature of the signal transduction activated by sub-toxic amounts of glutamate and causing up-regulation of bcl-2, as well as the nature of other death and survival signals and their interplay, is not yet known.

The results of this study suggest that glutamate, and possibly also other mediators of toxicity in the form of physiological compounds in excess of their normal amounts, has a gradient of effects. A slight increase in concentration above the physiological apparently acti-

vates a signal-transduction pathway that triggers corrective machinery of self-repair and self-maintenance, which not only is not harmful but even protects the cells, at least transiently, from further toxicity, and not necessarily only from the toxicity mediated by glutamate. Such a signal-transduction pathway might be collectively termed "SOS machinery." This pathway might be triggered even when glutamate reaches the threshold of toxicity, in which case some other mechanism operating concomitantly might drive the cells towards death. The balance between the two mechanisms would determine whether the fate of the cells is immediate apoptosis or survival with increased susceptibility. By analogy, very high concentrations of glutamate might result in a loss of neurons via necrosis.

Taken together, these findings point to the existence of an endogenous, self-operated mechanism of neuroprotection, which is self-limited by an excitatory amino acid that displays excitotoxicity when its normal physiological concentration is exceeded. This might represent a prototype mechanism for the regulation of an endogenous compound that has a crucial physiological role on the one hand but is potentially detrimental on the other. The relatively wide range of glutamate concentrations between the physiologically optimal and the cytotoxic would allow us to choose a therapeutic concentration that is not only subtoxic, but may also help induce subsequent resistance to a potentially toxic increase in glutamate concentration. Thus, administration of glutamate at an appropriate dosage might trigger an adaptive mechanism employed by the organism as a safety measure to reduce susceptibility and increase resistance to potentially harmful effects. It might also provide a mechanism of self-repair that can stabilize the organism in cases of minor trauma or transient physiological increases in glutamate concentration. Our findings thus introduce not only a new concept in neuroprotection but possibly also a new approach to the development of neuroprotective strategies for the treatment of chronic and acute degenerative diseases.

REFERENCES

- ALIAGA, E., RAGE, F., BUSTOS, G., et al. (1998). BDNF gene transcripts in mesencephalic neurons and its differential regulation by NMDA. *Neuroreport* 9, 1959–1962.
- BRUNO, V., SCAPAGNINI, U., and CANONICO, P.L. (1993). Excitatory amino acids and neurotoxicity. *Funct. Neurol.* 8, 279–292.
- CAMINOS, E., BECKER, E., MARTIN-ZANCA, D., et al. (1999). Neurotrophins and their receptors in the tench retina during optic nerve regeneration. *J. Comp. Neurol.* 404, 321–331.

- CANALS, J.M., CHECA, N., MARCO, S., et al. (1999). The neurotrophin receptors trkA, trkB, and trkC are differentially regulated after excitotoxic lesion in rat striatum. *Brain Res. Mol. Brain Res.* **69**, 242–248.
- CHAO, M.V. (1994). The p75 neurotrophin receptor. *J. Neurobiol.* **25**, 1373–1385.
- COHEN, A., BRAY, G.M., and AGUAYO, A.J. (1994). Neurotrophin-4/5 (NT-4/5) increases adult rat retinal ganglion cell survival and neurite outgrowth *in vitro*. *J. Neurobiol.* **25**, 953–959.
- CONNAUGHTON, V.P., BEHAR, T.N., LIU, W.L., et al. (1999). Immunocytochemical localization of excitatory and inhibitory neurotransmitters in the zebrafish retina. *Vis. Neurosci.* **16**, 483–490.
- COYLE, J.T., and PUTTFARCKEN, P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689–695.
- DAVANGER, S., OTTERSEN, O.P., and STORM-MATHISEN, J. (1991). Glutamate, GABA, and glycine in the human retina: an immunocytochemical investigation. *J. Comp. Neurol.* **311**, 483–494.
- DREYER, E.B., and GROSSKREUTZ, C.L. (1997). Excitatory mechanisms in retinal ganglion cell death in primary open angle glaucoma (POAG). *Clin. Neurosci.* **4**, 270–273.
- DREYER, E.B., ZURAKOWSKI, D., SCHUMER, R.A., et al. (1996). Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. *Arch. Ophthalmol.* **114**, 299–305.
- FADEN, A.I., and SALZMAN, S. (1992). Pharmacological strategies in CNS trauma. *Trends Pharmacol. Sci.* **13**, 29–35.
- FADEN, A.I., IVANOVA, S.A., YAKOVLEV, A.G., et al. (1997). Neuroprotective effects of group III mGluR in traumatic neuronal injury. *J. Neurotrauma* **14**, 885–895.
- FINKBEINER, S. (2000). CREB couples neurotrophin signals to survival messages. *Neuron* **25**, 11–14.
- FINKBEINER, S., TAVAZOIE, S.F., MALORATSKY, A., et al. (1997). CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19**, 1031–1047.
- FRIEDMAN, W.J., and GREENE, L.A. (1999). Neurotrophin signaling via Trks and p75. *Exp. Cell Res.* **254**, 131–142.
- GARCIA, I., MARTINOU, I., TSUJIMOTO, Y., et al. (1992). Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. *Science* **258**, 302–304.
- GINTY, D.D., BONNI, A., and GREENBERG, M.E. (1994). Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* **77**, 713–725.
- GRABB, M.C., and CHOI, D.W. (1999). Ischemic tolerance in murine cortical cell culture: critical role for NMDA receptors. *J. Neurosci.* **19**, 1657–1662.
- HU, B., YIP, H.K., and SO, K.F. (1999). Expression of p75 neurotrophin receptor in the injured and regenerating rat retina. *Neuroreport* **10**, 1293–1297.
- JOHNSON, E.C., DEPPMEIER, L.M., WENTZIEN, S.K., et al. (2000). Chronology of optic nerve head and retinal responses to elevated intraocular pressure. *Invest. Ophthalmol. Vis. Sci.* **41**, 431–442.
- JOJICH, L., and POURCHO, R.G. (1996). Glutamate immunoreactivity in the cat retina: a quantitative study. *Vis. Neurosci.* **13**, 117–133.
- KANAI, Y., SMITH, C.P., and HEDIGER, M.A. (1993). A new family of neurotransmitter transporters: the high-affinity glutamate transporters. *FASEB J.* **7**, 1450–1459.
- KORSMEYER, S.J. (1999). BCL-2 gene family and the regulation of programmed cell death. *Cancer Res.* **59**, 1693s–1700s.
- LEVI MONTALCINI, R. (1987). The nerve growth factor 35 years later. *Science* **237**, 1154–1162.
- MAH, S.P., ZHONG, L.T., LIU, Y., et al. (1993). The protooncogene bcl-2 inhibits apoptosis in PC12 cells. *J. Neurochem.* **60**, 1183–1186.
- MARC, R.E., LIU, W.L., KALLONIATIS, M. et al. (1990). Patterns of glutamate immunoreactivity in the goldfish retina. *J. Neurosci.* **10**, 4006–4034.
- MARINI, A.M., RABIN, S.J., LIPSKY, R.H., et al. (1998). Activity-dependent release of brain-derived neurotrophic factor underlies the neuroprotective effects of *N*-methyl-D-aspartate. *J. Biol. Chem.* **273**, 29394–29399.
- MARSHALL, K.A., DANIEL, S.E., CAIRNS, N., et al. (1997). Upregulation of the anti-apoptotic protein Bcl-2 may be an early event in neurodegeneration: studies on Parkinson's and incidental Lewy body disease. *Biochem. Biophys. Res. Commun.* **240**, 84–87.
- MCDONNELL, T.J., BEHAM, A., SARKISS, M., et al. (1996). Importance of the Bcl-2 family in cell death regulation. *Experientia* **52**, 1008–1017.
- MUKHERJEE, P.K., DECOSTER, M.A., CAMPBELL, F.Z., et al. (1999). Glutamate receptor signaling interplay modulates stress-sensitive mitogen-activated protein kinases and neuronal cell death. *J. Biol. Chem.* **274**, 6493–6498.
- NAG, T.C., and WADHWA, S. (1999). Neurotrophin receptors (Trk A, Trk B, and Trk C) in the developing and adult human retina. *Brain Res. Dev. Brain Res.* **117**, 179–189.
- NAKAO, N., and BRUNDIN, P. (1998). Neurodegeneration and glutamate-induced oxidative stress. *Prog. Brain Res.* **116**, 245–263.
- OLNEY, J.W. (1994a). Excitatory transmitter neurotoxicity. *Neurobiol. Aging* **15**, 259–260.
- OLNEY, J.W. (1994b). New mechanisms of excitatory transmitter neurotoxicity. *J. Neural Transm. Suppl.* **43**, 47–51.

GLUTAMATE EVOKES SELF-PROTECTION IN RETINAL GANGLION CELLS

- PEASE, M.E., MCKINNON, S.J., QUIGLEY, H.A., et al. (2000). Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma. *Invest. Ophthalmol. Vis. Sci.* **41**, 764–774.
- PIZZI, M., FALLACARA, C., ARRIGHI, V., et al. (1993). Attenuation of excitatory amino acid toxicity by metabotropic glutamate receptor agonists and aniracetam in primary cultures of cerebellar granule cells. *J. Neurochem.* **61**, 683–689.
- PIZZI, M., CONSOLANDI, O., MEMO, M., et al. (1996). Activation of multiple metabotropic glutamate receptor subtypes prevents NMDA-induced excitotoxicity in rat hippocampal slices. *Eur. J. Neurosci.* **8**, 1516–1521.
- ROCHA, M., MARTINS, R.A., and LINDEN, R. (1999). Activation of NMDA receptors protects against glutamate neurotoxicity in the retina: evidence for the involvement of neurotrophins. *Brain Res.* **827**, 79–92.
- ROWLAND, L.P. (1994). Amyotrophic lateral sclerosis. *Curr. Opin. Neurol.* **7**, 310–315.
- SAGARA, Y., and SCHUBERT, D. (1998). The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J. Neurosci.* **18**, 6662–6671.
- SAKAI, T., YOSHITOSHI, T., KAWAGOE, M., et al. (1999). Expression of brain-derived neurotrophic factor gene in retina following vitreous tap. *Nippon Ganka Gakkai Zasshi.* **103**, 271–276.
- SAWAI, H., CLARKE, D.B., KITTLEROVA, P., et al. (1996). Brain-derived neurotrophic factor and neurotrophin-4/5 stimulate growth of axonal branches from regenerating retinal ganglion cells. *J. Neurosci.* **16**, 3887–3894.
- SENDTNER, M., HOLTMANN, B., KOLBECK, R., et al. (1992). Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature* **360**, 757–759.
- STADTMAN, E.R. (1990). Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic. Biol. Med.* **9**, 315–325. (For erratum, see *Free Radic. Biol. Med.* 1991;10:249.)
- SUCHER, N.J., LIPTON, S.A., and DREYER, E.B. (1997). Molecular basis of glutamate toxicity in retinal ganglion cells. *Vision Res.* **37**, 3483–3493.
- TSUKAHARA, T., IIHARA, K., HASHIMOTO, N., et al. (1998). Increases in levels of brain-derived neurotrophic factor mRNA and its promoters after transient forebrain ischemia in the rat brain. *Neurochem. Int.* **33**, 201–207.
- YAN, Q., ELLIOTT, J., and SNIDER, W.D. (1992). Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* **360**, 753–755.
- YOLE, E., and SCHWARTZ, M. (1998a). Degeneration of spared axons following partial white matter lesion: implications for optic nerve neuropathies. *Exp. Neurol.* **153**, 1–7.
- YOLE, E., and SCHWARTZ, M. (1998b). Elevation of intraocular glutamate levels in rats with partial lesion of the optic nerve. *Arch. Ophthalmol.* **116**, 906–910.
- ZHONG, L.T., SARAFIAN, T., KANE, D.J., et al. (1993). bcl-2 inhibits death of central neural cells induced by multiple agents. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4533–4537.

Address reprint requests to:
Michal Schwartz, Ph.D.
Department of Neurobiology
The Weizmann Institute of Science
76100 Rehovot, Israel

E-mail: bnschwar@weizmann.weizmann.ac.il